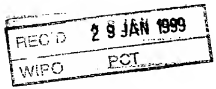
PCT/AU99/00004



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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 1280 for a patent by JOHNSON & JOHNSON RESEARCH PTY. LIMITED, COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION, GROUPE LIMAGRAIN PACIFIC PTY LIMITED and THE AUSTRALIAN NATIONAL UNIVERSITY filed on 9 January 1998.

WITNESS my hand this Twentieth
day of January 1999

KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

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AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION

FOR THE INVENTION ENTITLED:-

"METHOD FOR PLANT TRANSFORMATION"

The invention is described in the following statement:-

Technical Field

The invention relates to a method for the genetic transformation of plants, in particular poppies.

Background to the Invention

The importance of the plants of the poppy family, for example Papaver and Eschscholtzia species, as a commercial source of medicinal opiates and related compounds is well known, and requires little introduction. The demand for these plant products is high.

Suitable agricultural land for commercial poppy growing is limited. Poppies need fertile, free draining soil which is not overly acidic. To reduce the build up of disease in commercially grown poppies, crops must be grown with at least a three year rotation (e.g. at least two to three different crops should be grown in the soil before poppies are grown again). There are other limitations such as topography and availability of water for irrigation. Presently in some areas, the crop area is probably close to the sustainable level - if higher yield of poppy products are desired then it will be necessary to either shorten the rotation or expand the area under cultivation to include marginal soil types. It is expected that employing these less than desirable practices will impact on factors such as yield and quality and produce undesirable related outcomes such as soil erosion and so on.

The alkaloid content in harvested poppy straw in Tasmania, for example, is generally in the range of 1.2% to 2.7% on a dry weight basis. The financial return to the growers is calculated on the basis of the alkaloid content. Thus, high alkaloid content plants mean that the poppy industry can compete with alternative crops which might

potentially be grown in the same soils. High alkaloid content in the poppy crops makes the whole industry more competitive. Fewer hectares of crops would need to be grown to produce the same amount of alkaloid, and costs associated with harvesting, transport, storage, extraction and waste disposal would be reduced. Thus, high alkaloid producing poppies are highly desirable to growers, pharmaceutical companies and consumers of refined poppy products.

Conventional plant breeding has produced significant advances in poppy alkaloid contents over the last two decades. However, it appears that the amount of additional improvement possible through conventional breeding is limited.

Genetic transformation of poppies offers the opportunity to improve the alkaloid content of poppy crops and poppy straw. This could occur through a number of ways, including:

- enhancement of activity of enzymes at "bottlenecks" in the alkaloid synthetic pathway;
- blockage of undesirable "side reactions"; and
- blockage of the synthetic pathway so that certain desirable alkaloids accumulate (e.g. thebaine, codeine, oripavine etc.).

These types of improvement would thus allow the industry to continue to expand without increasing the area of crops grown. They would also introduce efficiencies throughout the production process.

As well as increasing the yield of desirable plant products, it is desirable to use related biotechnological procedures to introduce herbicide resistance into poppies. At present herbicide control of weeds in poppy crops is difficult and costly. Herbicides are

not developed specifically for poppies and the spectrum of weed control of any one herbicide is not very wide. Thus programs of herbicides are applied involving a number of different products tank-mixed and applied in sequence. Development of a herbicide resistant poppy will enable the use of a herbicide with a wider margin of crop safety, and a wider weed spectrum than currently available. The cost of such weed control is expected to be significantly less than presently involved.

Genetic transformation may also be used to introduce other genes into poppies to impart commercially desirable properties, for example. resistance to disease. resistance to acid soil and resistance to insects and other pests.

Despite the desirability of such transformations. it has so far proved difficult to produce viable transgenic poppies. Attempts using conventional methods to introduce specific gene sequences encoding for certain properties and subsequent regeneration of transgenic poppies with predictable properties have thus far been unsuccessful.

Thus there exists a need to develop a method for introducing genetic material into a plant which stably results in a plant which is viable and which possesses the desired traits.

It is an object of the present invention to overcome or ameliorate at least one or more of the abovementioned deficiencies in the prior art.

Summary of the Invention

Surprisingly, the present applicant has found that the unexpected rise in the pH of a tissue culture medium following the introduction of exogenous genetic material into plant tissue (e.g. a hypocotyl) is at least in part responsible for lack of success in regenerating poppy plants after transformation.

Throughout this specification, "type I callus" is translucent callus which can be colourless or brown, and is composed of large vacuolate cells. "Type II callus" is white to brownish opaque callus, composed of small cytoplasmic cells and having the capacity to form somatic embryos and meristemoids.

According to a first aspect, the invention consists in a method of producing a transgenic plant comprising the steps of :

1) transforming plant material with an exogenous genetic material;

2) culturing the transformed material in the presence of a buffering agent which prevents or reduces a rise in pH of the culture medium or plant tissue used for transformation; and

3) regenerating a transgenic plant.

Preferably the plant is an alkaloid producing poppy plant and even more preferably the plant is selected from the Papaver species or Eschscholtzia species. The most preferred species is *Papaver somniferum*.

Preferably the culture is derived from seeds, imbibed seeds or seedling parts of the plant. Preferably the culture is selected from the group comprising seed explant, seedling explant, type I callus, type II callus and somatic embryogenic callus.

Preferably the buffering agent is MES buffer, although in the light of the teaching of this disclosure those skilled in the art will be able to identify other buffers suitable for use in the method.

Preferably the exogenous genetic material is introduced into plant cells by a plant transformation agent, most preferably *Agrobacterium tumefaciens*. In another preferred

embodiment the exogenous material may be introduced using a mechanical method such as microparticle bombardment.

Preferably the exogenous genetic material encodes for an mRNA or protein that confers on the transgenic poppy a property selected from the group comprising: increased alkaloid yield relative to native alkaloid producing plant, increased herbicide resistance relative to native alkaloid producing plant, increased soil acidity tolerance relative to native alkaloid producing plant, increased disease resistance relative to native alkaloid producing plant, increased insect resistance relative to native alkaloid producing plant, increased growth rate relative to native alkaloid producing plant, improved flowering properties relative to native alkaloid producing plant and altered alkaloid proportions relative to native alkaloid producing plant. Most preferably the exogenous genetic material encodes for altered alkaloid proportions relative to native alkaloid producing plant. When the exogenous genetic material encodes herbicide resistance, preferably the herbicide resistance is Basta herbicide resistance, glyphosate resistance, bromoxynil resistance or sulfonylurea resistance.

Preferably the exogenous genetic material is comprised in a DNA construct based on the binary vector pPZP, most preferably pTAB101 with 35S 5':pat:35S 3'. In another preferred embodiment the binary vector is pBSF16.

According to a second aspect the invention consists in a transgenic plant prepared by the method of the first aspect.

Preferably the plant is an alkaloid producing poppy plant and even more preferably the plant is selected from the Papaver species or Eschscholtzia species. The most preferred species is *Papaver somniferum*.

Description of the Figures

Figure 1 Shows the plasmid pTAB101, which is a preferred vector for introducing exogenous genetic material in accordance with the present invention.

Figure 2 Shows pBSF16, which is an alternative binary vector for introducing exogenous genetic material in accordance with the present invention.

Best Method of Performing the Invention

The method comprises the use of a standard method of producing transgenic plants but with the additional improvement of adding MES buffer.

In most plants, the introduction of novel DNA is not accompanied by a rise in pH, however, this hitherto unexpected phenomena has been identified in poppy cultures. It has been observed by the applicants that there is an unexpected and rapid rise in the pH of tissue culture media derived from *Papaver somniferum*. This very rapid and substantial rise, for example, from pH 5.6 to pH>6.4 in the immediate area around a Type II callus in B5O medium within 30 minutes, rising ultimately to pH 8.7, has been identified as a major cause of poor growth and an inability to produce transgenic poppies.

The pat gene serves two purposes, as a selectable marker in vitro and as the herbicide resistance gene in the transgenic plant. As a selectable gene, it enables selection of transgenic cells in the culture using Basta herbicide or the active ingredient, glufosinate ammonium or phosphinothricin (also known as PPT). Those skilled in the art will know that alternative selectable genes could be employed such as those conferring hygromycin resistance, kanamycin resistance or spectinomycin resistance.

It will also be known to those skilled in the art that it is possible to introduce exogenous genetic material coding for more than one desirable property. For instance, the pBSF16 vector has three genes in the T-DNA: the bar gene conferring Basta (or PPT) resistance; the sunflower albumin gene, SF8g, which enables a novel sunflower seed albumin to accumulate in the seeds of the transgenic plant; the GUS reporter gene, which encodes β-glucuronidase and enables the detection of transgenic tissues.

Examples

Example 1. Plant material

The genotypes of *Papaver somniferum* used were C 046-3-5, C 058, C 060 and D 233 (Norman) obtained from Tasmanian Alkaloids. Seeds are surface sterilised by washing for 30-60 seconds in 70% ethanol then in 1%(w/v) sodium hypochlorite solution plus 1-2 drops of autoclaved Tween 20 or Triton X for 20 minutes with agitation. Seeds are rinsed three to four times in sterile distilled water or until no smell of bleach remains and placed on 90 x 25 mm Petri dishes containing B5O medium (see below). Dishes are sealed with Micropore tape and are usually stored at 4°C for 24 to 48 hours. Seeds are germinated at 24°C in a 16 hour light-8 hour dark cycle. Hypocotyls are excised from seedlings after 7-8 days of culture and are cut into 3-6 mm explants (usually 1-3 explants per seedling) and used in transformation experiments.

Example 2. Tissue culture media and conditions

All culture media consist of B5 macronutrients, micronutrients, iron salts and vitamins (Gamborg et al. 1968) and 20g/L sucrose. pH is adjusted with 1M KOH to pH 5.6, media is buffered with 10mM MES (2-[N-Morpholino]ethanesulfonic acid) and the gelling agent is 0.8% Sigma Agar. Growth regulators are added to media prior to autoclaving at 121°C for 20 minutes. B5O has no growth regulators and Callusing Medium (CM) has 1mg/L 2,4-D. Antibiotics are added after autoclaving and cooling to 55-65°C. Explant and type I callus cultures are grown in Petri dishes sealed with Micropore tape at 24°C. Type II callus and somatic embryos are cultured at 18°C.

Example 3. Bacterial strains and binary vectors

The disarmed *Agrobacterium tumefaciens* strains AGLO and AGL1 (Lazo et al., 1991) are used in transformation experiments. DNA constructs are based on the binary vector pPZP201 (Hajdukiewicz et al, 1994), e.g. pTAB101 (see Fig. 1) with 35S 5':pat:35S 3'. Agrobacterium strains are maintained in glycerol at -80°C or on LB agar plates plus appropriate selection at 4°C. Fresh cultures are grown overnight at 28°C in 10 mL MG broth (Garfinkle and Nester, 1980) without antibiotics. This Agrobacterium suspension is diluted to approximately $5x10^8$ cells mL^{-1} (OD600 = 0.25) for use in transformation experiments.

Example 4. Transformation and embryogenesis

Hypocotyls are excised from seedlings and immediately inoculated by immersion in liquid Agrobacterium culture for 10-15 minutes. Explants are then transferred directly to CM. After four to five days co-cultivation explants are washed in sterile distilled water, until the water is clear of Agrobacterium, blotted on sterile filter paper and transferred to CM containing 150 mg/L Timentin plus 10 mg/L PPT (phosphinothricin, the active ingredient of Basta herbicide). Explants are transferred to fresh CM at three weekly intervals. They initially produce friable brownish type I callus and may subsequently form small regions of very white, compact embryogenic callus (type II) by about 7-8 weeks culture.

Type II callus is transferred to B5O containing 150 mg/L Timentin plus 10 mg/L PPT and cultures are transferred to fresh medium every three weeks. Meristemoid/embryo development usually occurs after one or two periods on B5O medium and are seen from about 14-16 weeks total culture time.

Plantlet development from embryos is slow and may require a further 3 months in tissue culture before shoot and root growth is sufficient to ensure successful transplantation to soil.

Example 5. Importance of pH buffering

If the initial pH of the medium is 5.8 and MES is omitted, the pH of poppy cultures rapidly rises to pH 8.0 or higher. Fresh agar-solidified B5-based medium adjusted to pH 5.6 rose to pH>6.4 in the immediate area around type II callus within 30 mins. The inclusion of chlorophenol red in the medium was used to observe these localised increases in pH: the medium turns purple at pH6.4. The whole plate was pH>7 within 24 h. At the end of the culture period pH values were measured at 8.7. This rapid rise in pH results in very poor growth which is not compensated for by frequent changes of medium. The rapid rise was significantly delayed even by 2.5 mM MES, but 10 mM MES is preferred to adequately buffer the medium and support improved growth over the 3 week subculture period. Over 500 explants treated as above but on medium without MES failed to produce any transgenic shoots. At least three confirmed transgenic plants have been obtained from 1640 explants on MES buffered medium.

Table I

Importance of MES buffering

	Explants	Type II Calli	Embryos	Confirmed transgenic embryos or plants
-MES 2 (Expts.)	530	262	45	0
+MES 5 (Expts.)	1640	231	106	11 (>3 independent events)

References

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cells Res.* **50,** 151-158.

Garfinkle, D.J. and Nester, E.W. (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. *J. Bacteriol.* **144,** 732-743.

Hajdukiewicz, P., Svab, Z and Maliga, P. (1994). The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* 25, 989-994.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium. Bio/Technology*, **9**, 963-967.

DATED this 9th Day of January, 1998

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COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION,

GROUPE LIMAGRAIN PACIFIC PTY. LIMITED and

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Fellow Institute of Patent Attorneys of Australia
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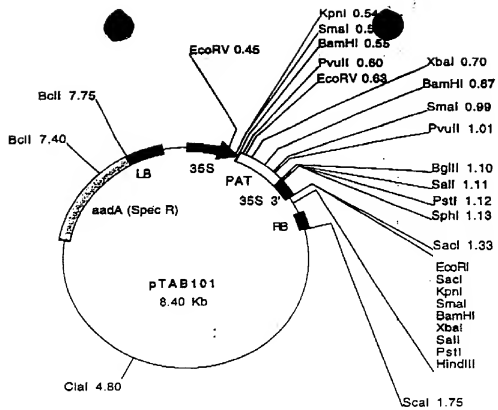
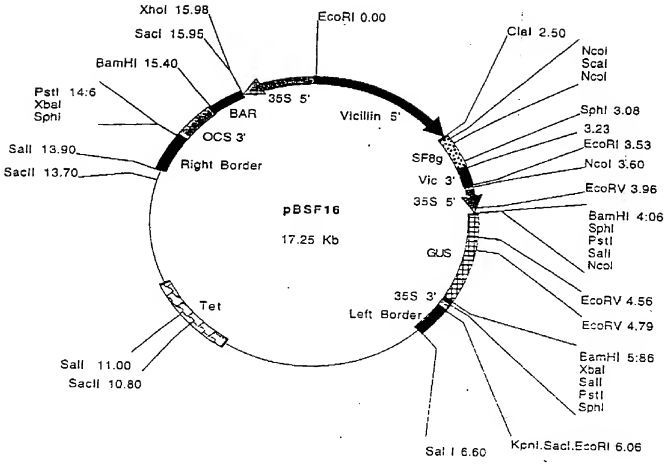
Fig 1



Plasmid name: pTAB101
Plasmid size: 8.40 kb
Constructed by: Linda Tabe
Construction date: 8.4.97
Comments/References: 35S-PAT gene from p35S-AcK cloned into EcoRI site of pPZP201 (Hajduklewicz et al., 1994 Plant Mol. Biol. 25: 989-994

FIG. 2



Plasmid name: pBSF16
Plasmid size: 17.25 kb
Constructed by: Linda Tabe
Construction date: ~ 30·4·92 II 159.
Comments/References: The region outside (and including) the T-DNA borders is from pGA472 (An et al 1985 EMBO 4:277).
The 35S-BAR-OCS construct is from pSLJ2011 (Jones et al 1992 Transgenic Res 1:285).